REMARKS

I. Claim Amendments

Claim 2 has been amended to more clearly define the invention and to place it in accordance with U.S. patent practice. Claims 3, 9, 11-17, 19-23, 25-26, 28-33, 47, 54, and 61 have been withdrawn from consideration pending reinstatement upon determination of allowable subject matter in the elected claims.

Claims 2, 4-8, 41 and 42 are currently under consideration by the Examiner.

The group R_1 in claim 2 has been amended by replacing "C_1-C_6 alkyl, substituted with one or more basic groups" with "C_4-C_6 alkyl, substituted with one or more basic groups".

Claim 2 has also been amended to remove NR_6CO from group X. Thus, after amendment the recitation of substituent X should read "X represents O, S, SO, $C(Z)_2$, N(Z), NR_6SO_2, SO_2NR_6 and $CONR_6$".

No new matter has been introduced by any amendment herein.

II. The claimed invention

The claimed invention is directed to pharmaceutical formulations of an inhibitor of carboxypeptidase U (CPU) and a thrombin inhibitor in admixture with a pharmaceutically acceptable adjuvant, diluent or carrier.

III. Claim rejections under 35 U.S.C. §§ 103

Claims 2, 4, 5, 8, 41, and 42 are rejected under 35 U.S.C. §103 as allegedly being unpatentable over Eisenbach-Schwartz in view of Watson.

The Examiner asserts that Eisenbach-Schwartz discloses that the dipeptide Arg-Cys can be used to treat various inflammatory disorders. Eisenbach-Schwartz, however, is acknowledged not to suggest combining this dipeptide with a thrombin inhibitor.

Furthermore, the Examiner cites Watson as disclosing that thrombin inhibitors are effective in treating various inflammatory conditions. Watson, however, is acknowledged not to disclose using cysteine derivatives to treat inflammatory disorders.

By Amendment of the definition of R_1 in claim 2, the dipeptide Arg-Cys, inter alia, has been excluded, and whether or not Eisenbach-Schwartz teaches the use of Arg-Cys and whether or not Watson teaches the use of thrombin inhibitors are moot.

Withdrawal of the alleged obviousness rejection of the claimed invention over Eisenbach-Schwartz in view of Watson is respectfully requested.

Claims 2, 4, 5, 8, 41, and 42 are rejected under 35 U.S.C. §103 as allegedly being unpatentable over Ondetti in view of Watson.

The Examiner states that "an artisan of ordinary skill may have been motivated to combine the compounds of Ondetti with the compounds of Watson for additive effects" (pages 8 and 9 of the Office Action mailed February 9, 2004).

The Examiner asserts that Ondetti discloses compounds, encompassed by instant claim 2, that are useful in treating cardiovascular conditions, inflammatory conditions and edema. Ondetti, however, is acknowledged not to suggest combining the disclosed compounds with thrombin inhibitors.

Furthermore, the Examiner cites Watson as disclosing that thrombin inhibitors can be used to treat various cardiovascular conditions, inflammation and edema. Watson, however, is

acknowledged not to suggest combining the thrombin inhibitors with the compounds of Ondetti.

By amendment of the definition of group X in claim 2, the compounds of Ondetti have been excluded and whether or not Ondetti teaches the use of the cited compounds and whether or not Watson teaches the use of thrombin inhibitors are moot.

Withdrawal of the alleged obviousness rejection of the claimed invention over Ondetti in view of Watson is respectfully requested.

Claims 2, 4, 5, 8, 41, and 42 are rejected under 35 U.S.C. §103 as allegedly being unpatentable over Eisenbach-Schwartz in view of Franson.

The Examiner cites Eisenbach-Schwartz for the same reasons and further asserts that Franson discloses compounds that inhibit thrombin-induced platelet aggregation, and at the same time, can be used to treat various inflammatory conditions. Franson however, is acknowledged not to disclose using cysteine derivatives to treat inflammatory disorders.

Again, by amendment of the definition of R_1 in claim 2, the dipeptide Arg-Cys, inter alia, has been excluded, and whether or not Eisenbach-Schwartz teaches the use of Arg-Cys and whether or not Franson teaches the cited inhibitory compounds and their use are moot.

Withdrawal of the alleged obviousness rejection of the claimed invention in view of Eisenbach-Schwartz and Franson is respectfully requested.

Furthermore, Applicants would like to address comments the Examiner made on pages 7 and 11 of the Office Action dated February 9, 2004 pertaining to the present invention.

The Examiner wrote: "The issue, however, is the extent to which the 'unexpected results' may extend to other thrombin

inhibitors which were never contemplated by applicants, or at least which were never disclosed in the specification. Other thrombin inhibitors may exhibit different pharmacokinetics/biodistribution than may be exhibited by inogatran or melagatran. Or the mechanism of thrombin inhibition may be different."

Applicants respectfully disagree with the Examiner for the following reasons: It is well known that thrombin, particularly in the presence of thrombomodulin, induces the activation of CPU from pro-CPU. Then, in turn, the degree of CPU activity determines the extent to which the clot lysis is inhibited.

It has been shown that inhibition of thrombin by a thrombin inhibitor results in less activation of CPU. For example, when thrombin is inhibited by argatroban, the clot lysis is enhanced due to decreased activation of CPU (please see enclosed article of Hashimoto et al., *Thrombosis and Haemostasis*, 2002, 87:110-113). In the same way, enhancement of clot lysis is also observed when thrombin is inhibited by hirudin (please see enclosed article of Latacha et al., *Journal of Thrombosis and Haemostasis*, 2003, 2: 128-134). Argatroban is a low molecular weight compound which inhibits thrombin directly at the active site whereas hirudin is a high molecular weight polypeptide that inhibits thrombin by interacting simultaneously with the active site and exosite I (please see enclosed article of Huntington et al., *TRENDS in Pharmacological Sciences*, 2003, Vol. 24, No. 11, pp. 589-595). Thus, inhibition of thrombin with either argatroban or hirudin results in enhancement of clot lysis due to decreased activation of CPU. This decrease of CPU activation is achieved regardless of the mechanism of inhibition of thrombin. It can also be pointed out that melagatran, for which the synergistic effect has been observed, and argatroban are

both active site inhibitors and bind to thrombin in essentially the same way (please see figure 2 in the above-cited Huntington article). Accordingly, the Examiner's assessment that the 'unexpected results' may extend to other thrombin inhibitors which were never contemplated by applicants nor disclosed in the specification is inappropriate.

Additionally, the Examiner stated on pages 7 and 11 of the aforementioned Office Action: "Further, if the artisan of ordinary skill is intent on treating cardiovascular disease, inflammation or oedema, one is unlikely to be concerned about the degree of inhibition of fibrin deposition in the lungs." However, Applicants only claim treatment of thrombosis and hypercoagulability. Therefore, the results presented in tables I-III are pertinent to the claimed subject matter. Moreover, it is irrelevant to the concept of patentability to distinguish the treatment of cardiovascular disease, inflammation or edema from the claimed treatment of thrombosis and hypercoagulability. The results presented by Applicants would be seen by the skilled artisan as unexpected regardless of said artisan's own particular research goals.

Claims 6 and 7 are objected to as being dependent on a rejected claim but have not been rejected for prior art reasons. Applicants submit that in view of their amendments and arguments, claims 6 and 7 are in condition for allowance.

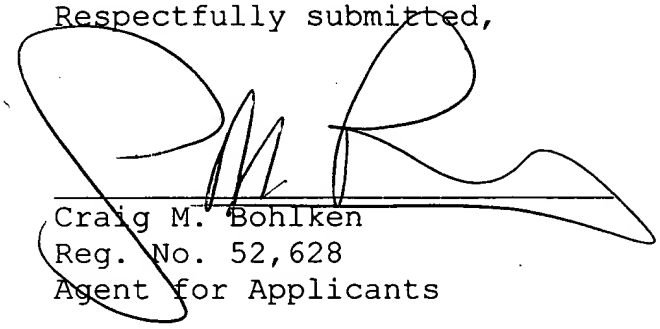
CONCLUSION

Applicants respectfully submit that the application is in condition for allowance, which action is earnestly solicited.

The Commissioner is hereby authorized to charge any fee which may be due in connection with this communication to Deposit Account No. 23-1703.

Dated: 08/09/2004

Respectfully submitted,



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Enclosures: three (3) references and a Notice of Appeal

Targeting thrombin – rational drug design from natural mechanisms

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It is difficult to overstate the medical importance of the serine protease thrombin. Thrombin is involved in many diverse processes, such as cell signaling and memory, but it is the crucial role that it plays in blood coagulation that commands the interest of the medical community. Thrombosis is the most common cause of death in the industrialized world and, whether through venous thromboembolism, myocardial infarction or stroke, ultimately involves the inappropriate activity of thrombin. The number and type of intrinsic and extrinsic natural mechanisms of targeting thrombin that have evolved validate thrombin as an important physiological target, and provide strategies to knock it out. The more we learn about the natural mechanisms that determine thrombin specificity the more likely we are to develop compounds that selectively alter thrombin activity. In this article, we review the natural mechanisms that regulate thrombin activity and novel approaches to inhibit thrombin based on these mechanisms.

Haemostasis, the balance between bleeding and thrombosis, is maintained by the localization and rapid amplification of coagulation proteases at the site of vascular injury and by mechanisms that inhibit these proteases elsewhere in the vasculature. Thrombin is central to the clotting arm of haemostasis and also plays a role in shutting down the coagulation cascade (Figure 1). How thrombin is both a pro-coagulant and anti-coagulant protease is a subject of much interest that is explained in part by regulatory cofactors such as thrombomodulin (TM). Thrombin is the product of an enzymatic amplification network in which inactive zymogen forms of many proteases and cofactors are activated by proteolytic cleavage. Thrombin cleaves fibrinogen into fibrin to create a fibrous plug. Thrombin also amplifies its own production through the activation of factor XI and cofactors V and VIII. In addition, thrombin activates the transglutaminase factor XIII that cross-links the polymerized fibrin plug, thus stabilizing the clot. During this process some thrombin remains bound to fibrin and becomes incorporated into and on the surface of the clot [1,2]. This ensures a pro-coagulant surface by protecting thrombin from inhibition by natural, circulating inhibitors [3]. Thrombin also plays a crucial role in the activation of platelets through the cleavage of the protease-activated receptors (PARs) on the platelet surface [4].

The clinical importance of regulating thrombin activity is highlighted by the use of anti-coagulant drugs to treat and prevent thrombosis. This is achieved by limiting functional prothrombin synthesis using warfarin, attenuating thrombin generation with low molecular weight heparin (LMWH) and by inhibiting formed thrombin with unfractionated heparin (UFH). Although these therapies are used widely, their anti-coagulant effects are often offset by unwanted side-effects, such as bleeding and heparin-induced thrombocytopenia. The ideal anti-thrombin drug would prevent thrombosis without causing excess bleeding, be orally available, inexpensive, reversible and not require monitoring. Rational drug design has already resulted in synthetic compounds that increasingly satisfy the profile of the ideal agent. In this article, we review the natural mechanisms that regulate thrombin activity, and selected approaches to inhibit thrombin based on these mechanisms.

Thrombin structure

The major breakthrough in understanding the functional properties of thrombin came in 1989 with the resolution of the first structure of human thrombin [5]. Since then > 150 structures of various states of thrombin bound to several substrates and inhibitors have been solved. We now have a detailed understanding of the structural organization of thrombin and the relationship between its unique structural features, cofactor, substrate and inhibitor binding, and activity. Figure 2a is a surface representation of thrombin that indicates the positions of the active site and exosites I and II. The thrombin active site is located in a deep canyon formed by the flanking loops, denoted the 60-insertion and γ-loops. The presence of these loops restricts access to the active site, thereby helping to determine substrate and inhibitor specificity. In addition, the position of the 60-insertion loop is sensitive to cofactor binding; thus, allostery plays a role in determining the activity of thrombin [6]. The specificity of thrombin is partially determined by the substrate sequence, and other determinants lie within exosites. Two basic exosites have been identified that are adjacent to the active site of thrombin, called anion-binding exosites I and II. Biochemical, mutagenic and structural studies have established the roles of exosites I and II [7]. Exosite I is a fibrin (ogen) recognition exosite that helps to determine the specificity for the substrate fibrinogen and is responsible for the sequestration of thrombin in fibrin clots. The endothelial cell-surface receptor TM also interacts with thrombin

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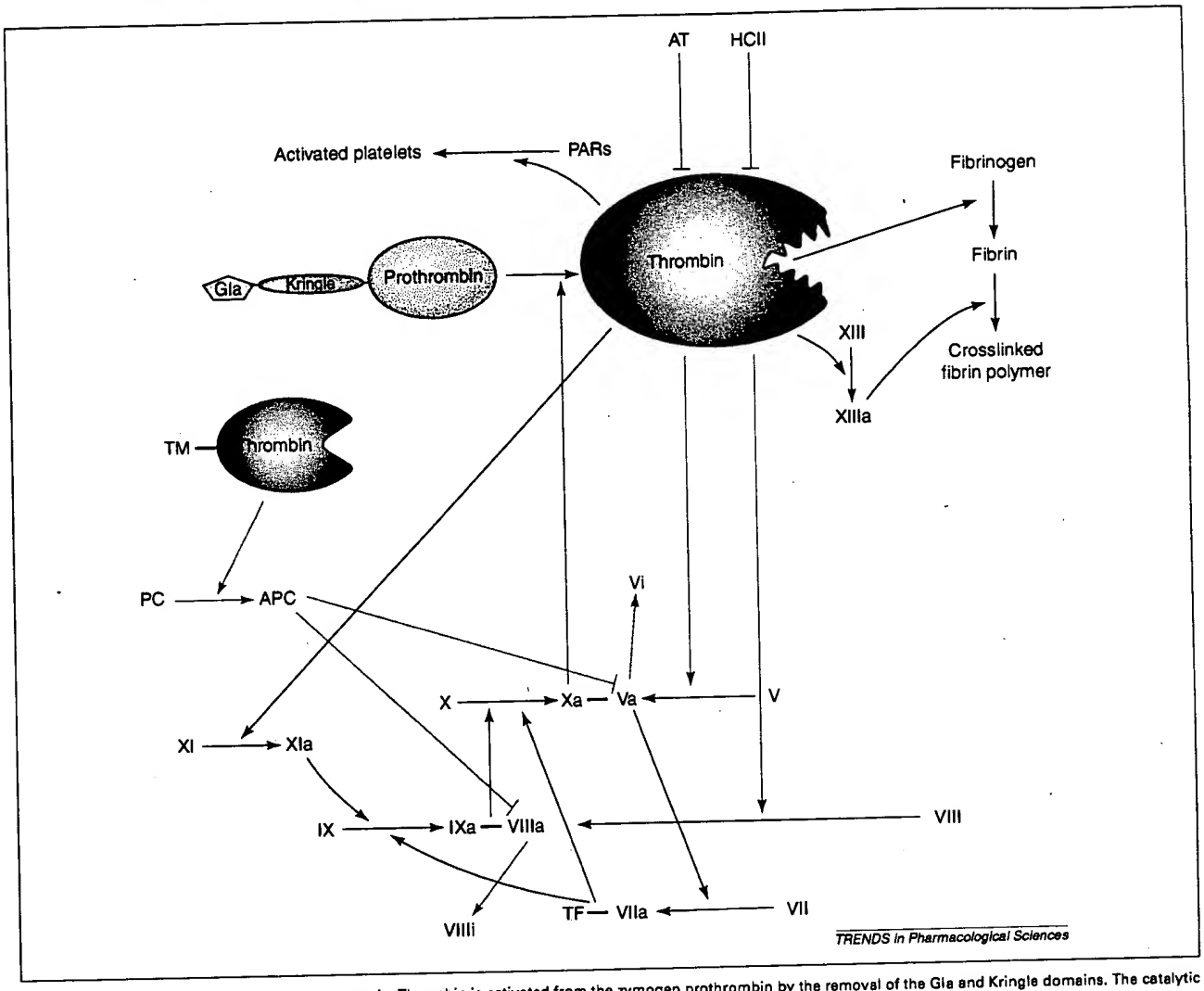

Figure 1. The central role of thrombin in haemostasis. Thrombin is activated from the zymogen prothrombin by the removal of the Gla and Kringle domains. The catalytic domain then interacts with its many substrates and cofactors. The pro-thrombotic role of thrombin includes the cleavage of figrinogen to fibrin, activation of factor XIII, which cross-links the fibrin polymers, and its own upregulation by activating factors XI, V, VIII and platelets. When thrombin binds to the endothelial cell receptor TM its activity is reversed from pro- to anti-coagulant (red pathway) through the cleavage and activation of protein C (PC). Activated protein C (APC) stops the generation of thrombin by inactivating factors Va and VIIIa. Thrombin is finally inhibited by the plasma serpins antithrombin (AT) and heparin cofactor II (HCII) that are resident on the heparin-like glycosaminoglycans that line the vasculature.

via exosite I, thereby blocking fibrinogen binding [8] and switching the substrate preference to the anti-coagulant protein C [9]. Exosite II is the glycosaminoglycan-binding site of thrombin [10]. Thus, the natural substrates, cofactors and inhibitors of thrombin have evolved to exploit exosites I and II. For example: fibrinogen [11,12] and PARs [13,14] interact with thrombin via exosite I; factor XI [15], factor XIII [16] and inhibitors antithrombin (AT) [10] and protease nexin 1 [17] use exosite II for either direct interactions or for glycosaminoglycan bridging; and factor V [18,19], factor VIII [20], TM [21,22] and heparin cofactor II (HCII) [23] can use both exosites. Thus, thrombin specificity is determined by multiple interactions, not solely through complementary active-site interactions. In this way, the activity of thrombin varies depending on where it is located and to what it is bound.

Physiological mechanisms of thrombin inhibition

Temporal and spatial control of thrombin activity is crucial for the prevention of thrombosis. Three factors are primarily responsible for limiting thrombin activity to the site of tissue damage: TM, AT and HCII. Although TM is not a true inhibitor of thrombin, it inhibits the pro-thrombotic activity of thrombin by altering its substrate specificity [24]. TM is a receptor that is exposed on the surface of intact endothelial cells. As mentioned above, TM binds to exosite I of thrombin to block the fibrinogen recognition site and prevent cleavage of fibrinogen (Figure 2b) [8]. It is not fully understood how binding of TM alters the substrate specificity of thrombin, but allostery and direct TM–protein C interactions have been suggested [25]. Recombinant, soluble TM is being developed as an anti-coagulant and is currently in Phase III clinical trials [26]. In many ways, the ideal anti-thrombin drug would behave like TM in reducing

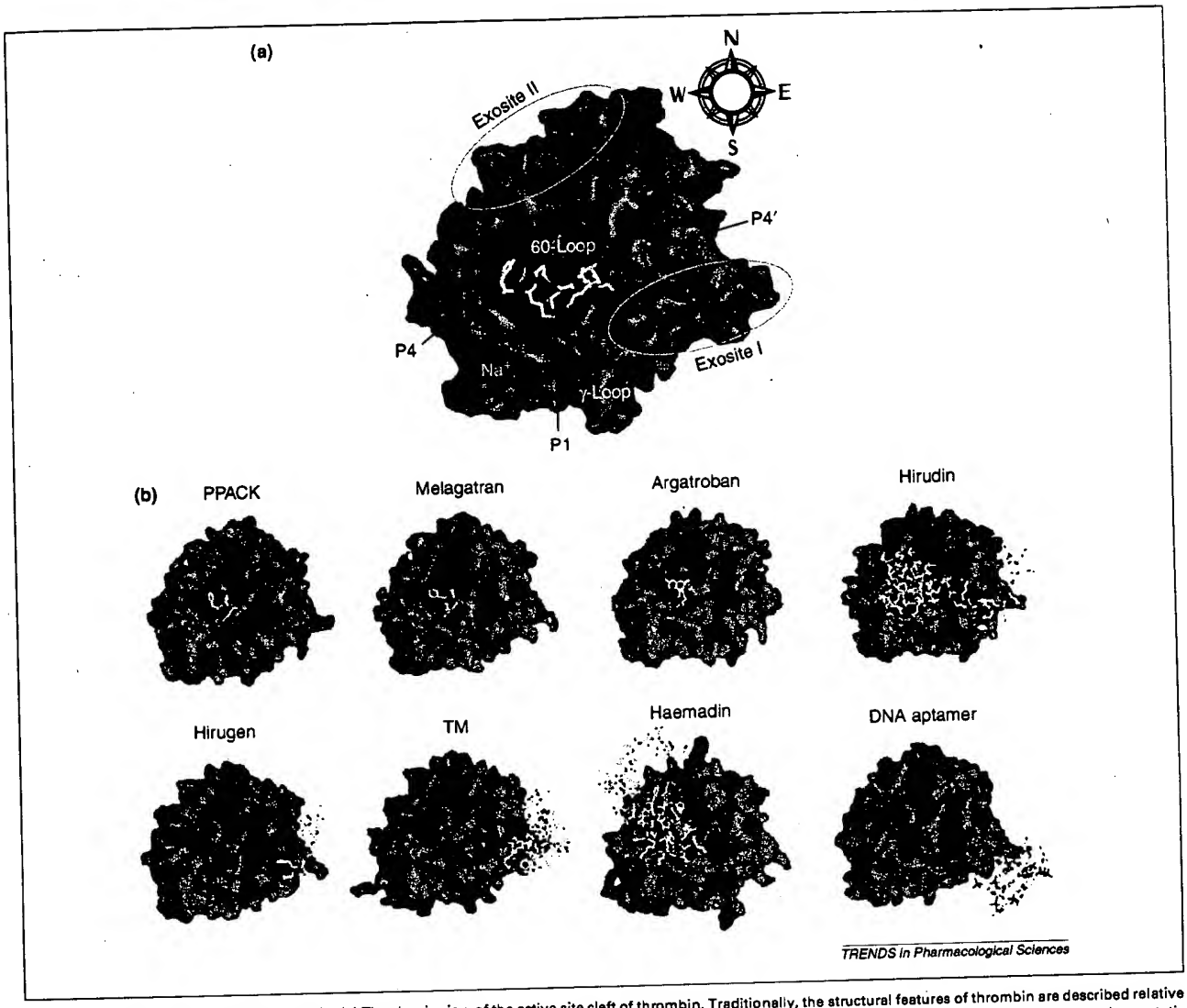

Figure 2. Surface representations of thrombin. (a) The classic view of the active site cleft of thrombin. Traditionally, the structural features of thrombin are described relative to the active site with the substrate running from West to East (N-terminal to C-terminal, P4 to P4′). The surface of thrombin is coloured according to the electrostatic potential (blue for positive charge and red for negative charge). The active site possesses an overall negative potential and prefers an arginine in the P1 position. The substrate peptide represented (rods) belongs to heparin cofactor II (HCII) and has a leucine in the P1 position. The non-primed side of the active site is a large hydrophobic cavity that prefers hydrophobic residues (P2, Pro; P3, Phe; and P4, Phe). Little is known about the primed side, but some specificity is derived from these interactions. The active site is buried deep in a canyon formed by the 60-loop and γ-insertion loop. Substrate and cofactor binding is often mediated by exosite interactions, and anion-binding exosites I and II are indicated. Thrombin activity is also affected by the binding of monovalent cations, and the Na^+ binding site is indicated. (b) Thrombin interactions with some active-site and exosite inhibitors. The surface of thrombin is shown in the same orientation as in (a), but coloured according to hydrophobic properties (green). The active-site inhibitors PPACK [(D-Phe)-Pro-Arg-chloromethylketone] (Protein Data Bank identifier 1PPB), melagatran (1K22) and argatroban (1DWC) superimpose well and occupy the substrate binding pockets from S4 to S1. Hirudin (4HTC) interacts avidly with exosite I, but also covers the active site to obtain a femtomolar dissociation constant. The C-terminal tail of hirudin (hirugen, 1DWC) binds to exosite I. TM (1DX5) interacts via exosite I (only part of the structure is shown) and alters the preferred substrate of thrombin from fibrinogen to protein C. Haemadin (1E0F) is a thrombin inhibitor from a haematophageous insect that interacts with exosite II and the active site. DNA aptamer technology has also been exploited to derive thrombin inhibitors, in this case interacting with exosite I (1HUT).

the activity of thrombin towards fibrinogen while either maintaining or enhancing the anti-coagulant effects of thrombin through the cleavage of protein C.

The true inhibitors of thrombin in the circulation are the serpins AT and HCII [27]. AT inhibits many of the proteases in the coagulation cascade, including factors IXa, Xa and thrombin, whereas HCII appears to be specific for thrombin. Because blood coagulation is a highly regulated process, it is not surprising that the serpins that inhibit the coagulation proteases are also subject to tight regulation. AT and HCII are poor inhibitors of their targets in their native, circulating form and it is only through specific interactions with glycosaminoglycans (GAGs) such as heparin that AT and HCII become efficient inhibitors. The molecular mechanisms of AT and HCII activation are given in Figure 3. Although these mechanisms were once thought to be unrelated, recently solved crystallographic structures demonstrate close structural and functional homology [23]. The poor activity of the circulating forms of both AT and HCII is maintained by restriction of the reactive centre loop (RCL) through its partial incorporation into β-sheet A, and the heparin binding sites are nearly perfectly conserved. There are, however, some major differences in how protease specificity is determined.

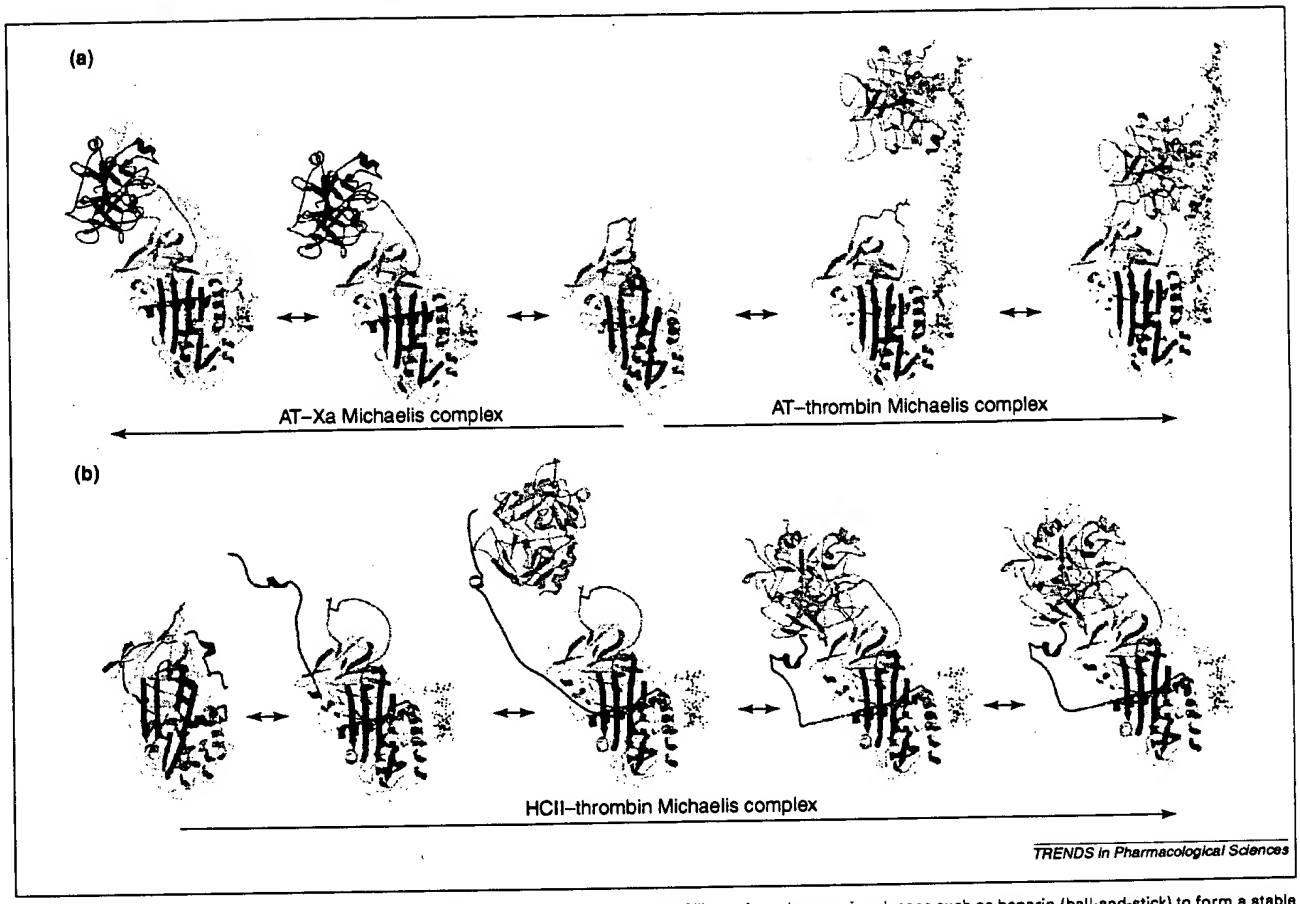

Figure 3. Thrombin inhibition by the serpins antithrombin (AT) and heparin cofactor II (HCII) requires glycosaminoglycans such as heparin (ball-and-stick) to form a stable Michaelis complex, and relies on active-site and exosite interactions. (a) AT (denoted by the ribbon diagram; reactive centre loop, yellow; β-sheet A, red; helix D, cyan; and P1 side-chain, red ball-and-stick) binds to a specific pentasaccharide sequence in heparin chains and undergoes an activating conformational change. The pentasaccharide itself significantly enhances the rate of factor Xa (magenta) inhibition (left); however, in the presence of Ca^{2+}, longer heparin chains (light grey ball-and-stick) can bridge factor Xa to AT, resulting in further enhancement [71]. Inhibition of thrombin (cyan, right) depends on the interaction between thrombin and heparin via exosite II on the same heparin chain occupied by AT. This requires >10 saccharide units on the non-reducing end of the specific pentasaccharide. (b) Inhibition of thrombin by HCII is more complex than the bridging mechanism employed by AT, and contains aspects of the AT–factor Xa mechanism. HCII (coloured as AT, but with the N-terminal hirudin-like tail in magenta) binds glycosaminoglycans in a similar fashion to AT and undergoes the same conformational change. The conformational change is associated with the release of the N-terminal tail, which then can recruit thrombin through hirudin-like interactions with the exosite I of thrombin. Thrombin then docks on the reactive centre loop to form extensive interactions with the body of HCII. In the presence of long-chain heparin the complex can be bridged through the interaction of heparin (light grey ball-and-stick) with exosite II of thrombin. In the case of AT and HCII, formation of the Michaelis complex is rate limiting, but is only the first step in the inhibitory mechanism. After formation of the acyl-enzyme intermediate, the protease is translocated to the opposite pole of the serpin, where it is crushed. Exosite I and the active site are destroyed, but exosite II remains intact.

AT binds with high affinity only to heparin and heparan sulfate because of the presence of a specific pentasaccharide sequence in a small percentage of these GAGs. Binding to the pentasaccharide induces a conformational change in AT that improves the affinity for heparin and frees the RCL for interaction with protease [28]. This conformational change has little effect on the rate of thrombin inhibition, but causes a 300-fold increase in the rate of factor Xa inhibition [29]. Thrombin inhibition by AT requires a longer heparin chain to allow the bridging of AT and thrombin through simultaneous binding to the same heparin molecule [30]. The thrombin-binding site for heparin is in the more basic exosite [10], exosite II, and requires at least six saccharide units. However, binding is not sequence specific and is of low affinity [31]. It is envisaged that AT saturates the high-affinity sites on the heparan sulfate molecules that line the microvasculature until thrombin diffuses from the site of tissue damage and encounters prebound AT [27].

It is possible that HCII behaves similarly but without the exquisite GAG specificity of AT. In fact, HCII can be activated by many GAGs including heparin, heparan sulfate and dermatan sulfate, but the binding affinity in all cases is ~1000-fold weaker than AT for its specific heparin sequence [32]. It has been proposed that HCII might have a role outside the circulation because it can be activated by GAGs in other tissues [33]. Adding to the speculation that the main physiological role of HCII lies outside the plasma is the limited importance of HCII in coagulation. Whereas AT deficiency is highly associated with venous thrombosis [34] and AT knockout is embryonic lethal in mice [35], HCII deficiency is not clearly associated with thrombosis [36] and HCII knockout mice are healthy [37]. However, HCII knockout mice have a propensity to arterial thrombotic occlusion. Whether the main role of HCII is to inhibit thrombin in the circulation or elsewhere, it is clear that HCII is a specific inhibitor of thrombin. Its specificity is conferred by an acidic N-terminal tail of ~80

amino acids that interacts with thrombin via exosite I [38]. Exosite II might also be involved when the activating GAG can bind to thrombin, thus leading to a double bridge. Therefore, the two circulating inhibitors of thrombin, AT and HCII, exploit unique properties of the anion binding exosites of thrombin to enhance specificity and ensure that coagulation is limited to the site of vascular injury.

Heparin therapy

Heparin has long been the anti-coagulant drug of choice for the prevention and treatment of thrombosis, and sales of heparin derivatives exceed 2 billion dollars per year [39]. Heparin is obtained in large amounts from the intestine of pigs, and is highly sulfated and heterogeneous. UFH ranges in size between 3000–30 000 Da with an average molecular weight of ~15 000 Da (~45 saccharide units). Approximately one-in-three heparin chains contains a copy of the AT-specific pentasaccharide, and these high-affinity sites account for the anti-coagulant properties of heparin preparations. Although still used, UFH has many drawbacks as a drug. UFH interacts with several proteins in the circulation, which reduces its bioavailability and can lead to heparin-induced thrombocytopenia (HIT) [40,41]. For these reasons heparin has been fragmented through either chemical or enzymatic depolymerization to yield LMWHs. There are many varieties of LMWHs, but all are heterogeneous with molecular weights that range from 2000 to 10 000 Da (6–30 saccharide units) [39]. The reduction in size, however, is associated with a reduction in the ability to inhibit thrombin. Most LMWHs primarily catalyze the inhibition of factor Xa by AT and, thus, inhibit the formation of thrombin. Even when sufficiently long to bridge AT and thrombin, therapeutic heparin is still limited to catalyzing the AT-dependent inhibition of fluid-phase thrombin. Thrombin bound to the fibrin clot is protected from heparin-induced inhibition because of a ternary complex between thrombin, fibrin and heparin [42].

Current preparations of heparin have little or no effect on the activity of HCII because HCII binds heparin ~1000 times more weakly than does AT. HCII is, thus, an untapped source of anti-thrombin activity that several reports suggest is uniquely capable of inhibiting both fibrin-bound and clot-bound thrombin [3,43,44]. Although thrombin is bound to fibrin via exosite I, the fibrin-binding site does not overlap with the binding site of the acidic tail of HCII [23]. Thus, specific activators of HCII would promote the inhibition of both fluid-phase and solid-phase thrombin. Danaparoid (Orgaran®) is a mixture of low molecular weight heparan, dermatan and chondroitin sulfates that is designed to activate AT against factor Xa, and HCII against thrombin [45]. Although licensed for the prevention of peri-operative deep vein thrombosis and the treatment of thromboembolic disease in patients with heparin-induced thrombocytopenia [41], it is unclear how much, if any, of the therapeutic effect is mediated via HCII. Another approach is to destroy the AT-binding sites on heparin through periodate oxidation, so that higher doses can be administered in an attempt to activate HCII. The resulting LMWH, Vasoflux™, is effective at inhibiting soluble and fibrin-bound thrombin [46]. However, preliminary clinical trials indicate that Vasoflux is no better than UFH and causes bleeding at the doses tested [47]. In addition, it is possible that the anti-thrombotic properties of Vasoflux could be independent of AT and HCII [48]. A similar dermatan-sulfate derivative, desmin, is being evaluated as a specific HCII activator [49] but, again, its activity might be independent of HCII [50]. More work needs to be done to tap the potential anti-thrombin activity of circulating HCII.

Another way to improve heparin therapy is to remove the heterogeneity of the preparations. The only way to obtain large amounts of pure, homogeneous AT-specific heparin is by chemical synthesis. Sanofi-Synthelabo (http://www.sanofi-synthelabo.com), in collaboration with Organon (http://www.organon.com/), has developed a synthetic pentasaccharide called fondaparinux [51] (Arixtra®) that has 100% bioavailability and a 17-h half-life, and specifically stimulates the AT-mediated inhibition of factor Xa. The crystal structure of the pentasaccharide–AT complex has been solved [28] and reveals, among other things, the orientation of the pentasaccharide on AT. This structure shows clearly that an effective AT–heparin–thrombin complex requires an extension on the non-reducing end of the pentasaccharide. Subsequent studies prove that addition of a six-unit thrombin-binding oligosaccharide on the non-reducing end, spaced by a linker of at least four sugar units (15 saccharide units in total) is required to activate AT towards thrombin [52]. The resulting molecule is effective against both factor Xa and thrombin, and has a similar bioavailability and half-life to fondaparinux. Although it has been suggested that AT is unable to inhibit clot-bound thrombin when activated by heparin, these observations are based on the administration of UFH, of which only a small fraction contains the AT-consensus pentasaccharide. The model that accounts for how the presence of heparin protects thrombin from inhibition by AT relies on the presence of excess heparin, uncomplexed by AT. A recent study has shown that if heparin is cross-linked covalently to AT, AT inhibits fibrin-bound thrombin [53]. This indicates that the new generation of synthetic heparins will be capable of inhibiting soluble thrombin and thrombin bound to the surface of either nascent or fibrinolyzed clots.

Direct thrombin inhibitors

Blood-sucking animals, haematophages, require a free flow of blood after piercing the blood vessel. Because of the central role of thrombin in clotting, the strategy adopted by most blood-suckers is to inhibit thrombin by introducing small, multivalent proteins [54]. There are several examples of crystal structures of thrombin bound to such protein inhibitors deposited in the Protein Data Bank (http://www.rcsb.org/pdb/), including 4HTC (hirudin) [55], 1THR [56], 1E0F (haemadin) [57], 1EOU, 1TBR [58], 1TOC [59], 1AVG [60] (reviewed in [61]). The best characterized and most advanced as a drug is hirudin. Hirudin is a 65-amino-acid polypeptide isolated originally from the medicinal leech (*Hirudo medicinalis*) that inhibits thrombin by interacting simultaneously with the active site and exosite I (Figure 2b) [62]. Although the complex is theoretically reversible, a dissociation constant in the range of 10^{-14} M renders it practically irreversible [63]. Recombinant molecular biology techniques

were required to exploit knowledge of how hirudin works towards the development of drugs. Recombinant hirudin (Lepirudin) is currently approved for the treatment of HIT. The discovery of the multivalent mechanism by which hirudin inhibits thrombin, coupled with a detailed structural understanding of how thrombin binds substrate-based inhibitors, has led to the development of peptide derivatives that mimic the active-site interactions of a substrate and the exosite I interactions of the C-terminal peptide of hirudin (hirugen) [61,64]. What distinguishes the several multivalent hirudin homologues (hirulogs) is the composition of the linker region between the two functional domains, which result in binding constants that range from 0.09 to 72 nM. Unfortunately, this high-affinity binding could be the reason for the narrow therapeutic window observed in clinical trials [65].

Since the publication of the first crystallographic structure of thrombin in 1989 much effort has been invested in the design of specific, low-molecular-weight, direct thrombin inhibitors, based on the principles of substrate recognition. Thrombin was crystallized after reaction with the peptide chloromethyl ketone derivative, (D-Phe)-Pro-Arg-chloromethylketone (PPACK). PPACK forms covalent bonds with both Ser195 and His57 in the active site of thrombin, but otherwise binds in a substrate-like manner (Figure 2b). The binding of PPACK, which has been described in detail, formed the basis of much of the subsequent rational drug design [66]. Indeed, melagatran, the most promising small inhibitor of thrombin under development, superimposes almost perfectly on the Phe-Pro-Arg of the original thrombin structure (Figure 2b), and the prodrug form ximelagatran is the only orally available direct thrombin inhibitor in Phase III clinical trials. An arginine-based compound, argatroban, is approved in the USA and Canada for the treatment and prevention of HIT [41]. Although not considered a tripeptide mimetic, the functional groups of argatroban fit well into the S1-, S2- and aryl-binding pockets.

Future directions

One of the most exciting achievements in directed thrombin inhibition is the development of ximelagatran, the orally available prodrug form of melagatran. One obvious strategy for improving the specificity of such active-site inhibitors is to include residues C-terminal to P1 (P′ side of the active site or 'East' in Figure 2a). The primed side runs into exosite I and determines the substrate specificity of fibrinogen. The difficulty in developing drugs based on P′ interactions is that until recently there has been no structural information on these interactions. Although peptide library screens indicated that much of the specificity was determined by P′ interactions [67], these have only been demonstrated crystallographically with the recent structure of catalytically inactive recombinant thrombin (S195A) bound to HCII [23]. Perhaps, in the future, mimetics similar to melagatran might include moieties designed to take advantage of sites C-terminal of the P1 residue.

Another promising approach is the development of new glycosaminoglycans. Although not yet on the market, bifunctional synthetic heparins that have a thrombin-binding site on the non-reducing end of the AT-specific pentasaccharide achieve optimal AT activation towards factor Xa and thrombin inhibition. We predict that they will inhibit both fluid-phase and solid-phase thrombin and be effective in the prophylaxis and treatment of many thrombotic states. The next step in the development of GAG drugs must be the discovery of specific activators of HCII. Identifying a natural GAG that contains a high proportion of a specific HCII-binding sequence will make HCII a viable drug target. Such a sequence has not yet been identified. A hexasaccharide has been isolated from dermatan sulfate based on HCII affinity, but this is in a very low percentage of chains and its affinity has not been demonstrated to be significantly higher than the background sequence [68]. As yet, no structure has been determined for HCII bound (however weakly) to a GAG. Once this has been achieved it might be possible to design oligosaccharides that have improved affinity and specificity for HCII. Another approach to improve heparin therapy is the development of orally available heparin through formulation of UFH and LMWH with carrier molecules SNAC {sodium N-[8-(2-hydroxybenzoyl)amino] caprylate} [69] and DOCA (deoxycholic acid) [70]. Early indications are that such formulations significantly improve the oral availability of heparin and have an anti-coagulant effect.

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Enhancement of Endogenous Plasminogen Activator-induced Thrombolysis by Argatroban and APC and Its Control by TAFI, Measured in An Arterial Thrombolysis Model *In Vivo* Using Rat Mesenteric Arterioles

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Keywords

Endogenous thrombolysis, fibrinolysis, thrombin, TAFI

Summary

Recent *in vitro* studies have demonstrated that thrombin inhibits fibrinolysis through thrombin-activatable fibrinolysis inhibitor (TAFI, plasma procarboxypeptidase B). We have recently shown that endogenous fibrinolysis *in vivo* is enhanced by activated protein C (APC) and the selective thrombin inhibitor, argatroban. The aim of the present study was to examine the role of TAFI in these fibrinolytic mechanisms *in vivo* using purified porcine pancreatic carboxypeptidase B (PPCPB) and a specific TAFIa inhibitor, potato tuber carboxypeptidase B inhibitor (PTCI) in a newly established arterial thrombolysis model. Non-occlusive, mural, platelet-rich thrombi were formed by helium-neon laser irradiation in rat mesenteric arterioles and thrombus size was measured by computerised image analysis. We confirmed that endogenous thrombolysis was enhanced by argatroban (2.0 mg/4 ml/kg/h) or APC (1.62 mg/2.31 ml/kg). PTCI (5.0 mg/2 ml/kg) also accelerated endogenous thrombolysis. PPCPB (3.5 mg/2 ml/kg) inhibited thrombolysis in the absence and presence of argatroban or APC. PTCI tended to further promote APC-induced thrombolysis but the differences did not reach statistical significance. The present findings were in keeping with the results of earlier studies and demonstrated that arterial, platelet-rich thrombi *in vivo* are degraded by naturally generated plasminogen activators. TAFI may play a significant role in the control of these mechanisms.

Introduction

Platelets are believed to play a central role in the development of acute arterial thrombi *in vivo* and subsequent fibrin formation is thought to consolidate the thrombus structure. Thrombin is a physiologically important agonist of platelet activation and is the primary enzyme responsible for the conversion of fibrinogen to fibrin. It seems likely, therefore, that thrombin plays a critically important role in arterial thrombogenesis (1-6).

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Recent studies *in vitro* using exogenous, tissue plasminogen activator (tPA), have demonstrated that thrombin governs not only fibrin formation but also fibrinolysis (7-13). Thrombin activates a TAFI. Activated TAFI (TAFIa, plasma carboxypeptidase B) cleaves the plasminogen binding site on fibrin resulting in lysis-resistant fibrin (10).

It has been demonstrated that TAFI is involved in thrombosis and PA-induced thrombolysis *in vivo* (11-13). In addition, neutralisation of Factor XI has been shown to enhance endogenous thrombolysis (14). In these experiments, however, the involvement of TAFI was studied using venous thrombi (13, 14). Furthermore, endogenous thrombolysis was not evident in an arterial model (11). We have previously demonstrated that the highly specific thrombin inhibitor, argatroban, enhanced exogenous plasminogen activator (PA)-induced thrombolysis of platelet-rich thrombi formed in rat mesenteric venules (15, 16). We have also extended these data and shown that argatroban or APC alone accelerates endogenous arterial thrombolysis *in vivo* (17, 18).

The aim of the present study was to examine the role of TAFI in the generation of endogenous arterial thrombolysis using purified PPCPB and a specific TAFIa inhibitor, PTCI, in a highly reproducible animal model of thrombolysis utilising rat mesenteric arterioles.

Materials and Methods

Animals and Agents

Animals: Male Wistar ST rats aged 8 weeks and weighing 230-270 g were obtained from SLC Co. Ltd. (Hamamatsu, Japan). The animals were fasted overnight prior to the thrombolytic experiments but allowed free access to water. All procedures were conducted in compliance with the Guiding Principles for the Care and Use of Animals in the Field of Physiological Sciences, published by the Physiological Society of Japan.

Agents: Argatroban (Novastan, 0.5 mg/ml) was purchased from Mitsubishi Tokyo Pharmaceutical Co. Ltd. (Tokyo, Japan) and diluted in physiological saline. APC purified from human plasma (3.5 mg/ml) was donated from Chemo-sero-therapeutic Research Institute (Kumamoto, Japan) and was stored at –80° C until use. It was dissolved and diluted in 20 mM citrate/0.7% NaCl/0.5% glycine (pH 7.0). PPCPB (protease inhibitor treated and affinity purified; 125-250 U/mg protein) was purchased from Sigma and was stored at –80° C. PTCI was purchased from Calbiochem (La Jolla, USA) and was stored at –80° C. The PPCPB and PTCI were dissolved and diluted in physiological saline immediately prior to use.

Helium-neon Laser-induced Thrombosis

Microvascular thrombi were produced in rat mesenteric arterioles using a slight modification of the method described previously (19, 20). Briefly, the animals were anaesthetised with nembutal (70 mg/kg, i. m.), and polyethylene cannulae (inner diameter 0.58 mm, outer diameter 0.97 mm, Becton Dickinson, USA) were inserted into both femoral veins. The mesentery was exposed and secured flat in Tyrode solution at 37° C on the stage of an operating microscope (Olympus BH-2). After 30 min Evans blue (14.2 mg/kg) was injected through one of the femoral vein cannulae and arterioles (outer diameter 40-50 μm were irradiated with a He-Ne laser beam at the midpoint between the internal vessel wall and the centreline of the lumen. The power and diameter of the laser spot at the focal plane were 15 mW and 15 μm, respectively. Irradiation for 2 s was repeated at 15-second intervals until the extent of the thrombus reached 90% of the lumen diameter. The thrombus was then allowed to stabilise for 10 min before the test agents were administered through the opposite femoral vein. Argatroban (0.67-2.0 mg/4.0 ml/kg/h) was continuously infused for 60 min. APC (0.54-1.62 mg/2.31 ml/kg), PPCPB (5.0 mg/kg/20 ml) and PTCI (3.15 mg/kg/2 ml) were given as bolus injections.

Computerised Image Analysis of Thrombolysis

The process of thrombolysis was continuously recorded on a videotape recorder. Subsequently, images at fixed time intervals were transferred to a personal computer and were analysed by Image Analyst software (Automatix, USA). The method used to calculate the dimensions of the thrombi is shown in Fig. 1. The consolidated thrombus within the vessel lumen was enclosed by a box. The grey scale threshold level was set to delineate the thrombus and the thrombus area was measured. Thrombus size was calculated by multiplying the area by the mean grey scale value. Thrombus size during thrombolysis was expressed relative to that of the thrombus immediately before agent administration.

Statistical Analysis

The results were analysed by multiple repeated ANOVA followed by Duncan post hoc test and were expressed as mean + SEM.

Results

Effect of Porcine Pancreatic Carboxypeptidase B and Potato Tuber Carboxypeptidase B Inhibitor on Argatroban-enhanced Endogenous Thrombolysis

Thrombus dissolution was monitored for 60 min during the continuous infusion of argatroban or saline and after the injection of test substances (Fig. 2). The rate of endogenous thrombolysis is illustrated by a temporal reduction in relative thrombus size in the control animals

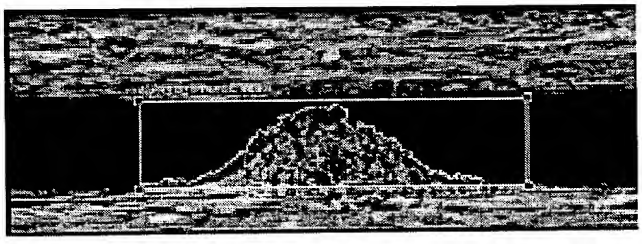

Fig. 1 Evaluation of thrombus size using Image Analyst software. The thrombus within the lumen of the arteriole was enclosed in a rectangular box and a threshold level defining the thrombus area was set on a grey scale. The extent of thrombolysis was calculated from the following formula: An × Gn/Ao × Go, where Ao is the thrombus area immediately before infusion of the test agents (time 0) and An the area during thrombolysis. Go is the grey average at time 0 and Gn during thrombolysis

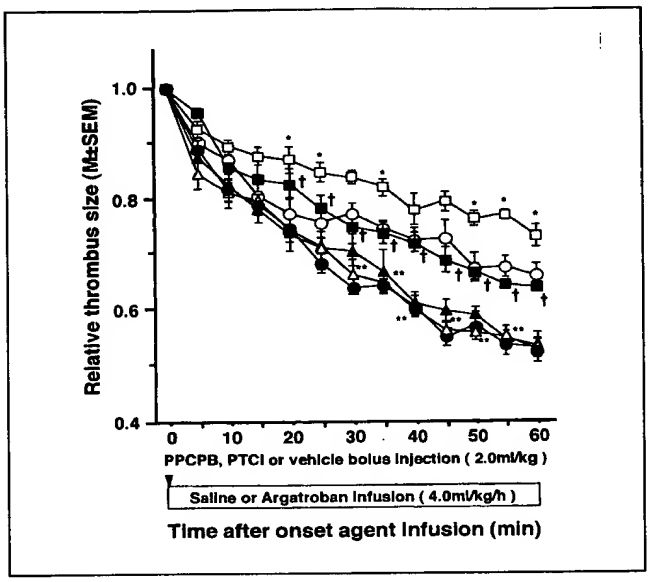

Fig. 2 Inhibition of argatroban-induced thrombolysis by PPCPB and enhancement of the endogenous thrombolysis by PTCI. ●: Argatroban + vehicle; □: saline + PPCPB; △: saline + PTCI; ■: argatroban + PPCPB; ▲: argatroban + PTCI; ○: saline + vehicle (control). n = 6~8 rats in each group. *: $p < 0.05$, **: $p < 0.01$ versus control and †: $p < 0.01$ versus argatroban + vehicle group in each time interval

continuously infused with saline in the absence of any other additive. Argatroban at a dose of 2.0 mg/kg/h increased the rate of endogenous thrombolysis compared with that seen in control animals. In contrast, PPCPB at a dose of 5.0 mg/kg reduced the basal rate of thrombolysis. Furthermore, PPCPB given with argatroban neutralised the accelerating effect of the thrombin inhibitor. In addition, PTCI at a dose of 3.15 mg/ kg enhanced endogenous thrombolysis. PTCI given concomitantly with argatroban, however, did not increase the rate of thrombolysis above that observed with argatroban or PTCI alone.

Effect of Porcine Pancreatic Carboxypeptidase B and Potato Tuber Carboxypeptidase B Inhibitor on APC-enhanced Endogenous Thrombolysis

Thrombus dissolution was monitored for 60 min after bolus injections of APC and test substances (Fig. 3). As in Fig. 2, the rate of endogenous thrombolysis is illustrated by a steady reduction in relative thrombus size in the control animals injected with saline alone. APC at a dose of 1.62 mg/kg increased this rate of natural thrombolysis. Again in this second series of experiments, PPCPB alone, at a dose of 5.0 mg/ kg, inhibited endogenous thrombolysis and PPCPB injected with APC neutralised the accelerating effects of the APC. PTCI alone enhanced endogenous thrombolysis and in these instances concomitant administration of APC and PTCI tended to increase the rate of thrombolysis above that observed with APC or PTCI alone, although the differences did not reach statistical significance. The reasons for the lack of statistical significance in this series of experiments are being investigated further, but may be due to differences between batches of animals.

Discussion

A variety of animal models have been described for the investigation of thrombolytic mechanisms *in vivo* (11-18, 21-23). Protocols designed

for this purpose need to be highly sensitive and reproducible, especially in studies of endogenous fibrinolysis where the intravascular responses to naturally generated plasminogen activators (PAs) may be considerably weaker than those evoked by exogenously administered PAs such as streptokinase. In the present study we have expanded our previous platelet-rich thrombolysis model (15, 16) to quantitate thrombolytic mechanisms in the arterial circulation using sophisticated image analysis software, Image Analyst (Automatix).

In this model thrombus formation was induced by helium-neon (He-Ne) laser irradiation in the presence of Evans blue. The precise mechanisms of thrombogenesis in these circumstances remain uncertain, but it is believed that Evans blue converts laser energy to heat resulting in disturbances in endothelial integrity and subsequent thrombosis (19, 20). We (24) and others (25) have shown that thrombi formed in this way are primarily composed of platelets, which adhere and aggregate on morphologically uninjured endothelial cells. The present model differs from several other models, therefore, in which platelet-rich thrombosis is induced after traumatic endothelial denudation (21-23).

We confirmed that the He-Ne laser-induced thrombi were predominantly platelet-rich and fibrin was not detected under high magnification of transmission electron microscopy (17). We have speculated, however, that fibrin-related reactions were involved in thrombus formation (18), because dissolution of the thrombus was prevented by tranexamic acid (AMCA), which inhibits specifically plasminogen or plasmin binding to fibrin resulting in inhibition of fibrinolysis (26). The rate of endogenous thrombolysis was demonstrated by a reduction in relative thrombus mass of approximately 29-33% over 60 min in control animals given saline in the absence of any other additive. The breakdown products of the thrombus were removed by physiological blood flow. Our earlier studies showed that this rate of lysis was partly suppressed by AMCA (17, 18) and we have now demonstrated that exogenous PPCPB has a similar effect, indicating that reactions involving TAFIa as well as plasmin control this thrombolytic process.

The selective thrombin inhibitor, argatroban, enhanced the rate of endogenous thrombolysis and this enhancement was neutralised by concomitant administration of PPCPB. These results were again similar to our previous findings using AMCA (17, 18). Commercially available PPCPB was used as an alternative to TAFIa in these experiments. Although PPCPB is not identical to TAFIa or plasma carboxypeptidase B, they have similar substrate specificities and there are highly significant biochemical homologies between species (27, 28). Thus, our results suggested that both TAFIa and plasmin were implicated in the profibrinolytic effects of argatroban. In addition, administration of PTCI accelerated endogenous thrombolysis indicating that inhibition of naturally occurring TAFIa promoted fibrinolytic activity. The results were in keeping with other *in vitro* studies and confirmed a significant role for TAFIa in thrombolysis *in vivo*. However, in contrast to the present results, PTCI did not induce endogenous thrombolysis in an arterial (aortic) thrombolysis model after endothelial denudation and stenosis (12). The reasons for the differences are not entirely clear but may reflect differences in sensitivity of the *in vivo* models. Endogenous thrombolytic activity may be much weaker than that induced by exogenous plasminogen activators, and we used non-occlusive microthrombi formed in arterioles to improve the sensitivity of our technique. In addition, our experiments were performed in the absence of stasis. It may be that test substances are incorporated more effectively into developing thrombi in the presence of flow and thus, localised thrombolytic mechanisms may be enhanced to a greater extent than those in the absence of flow.

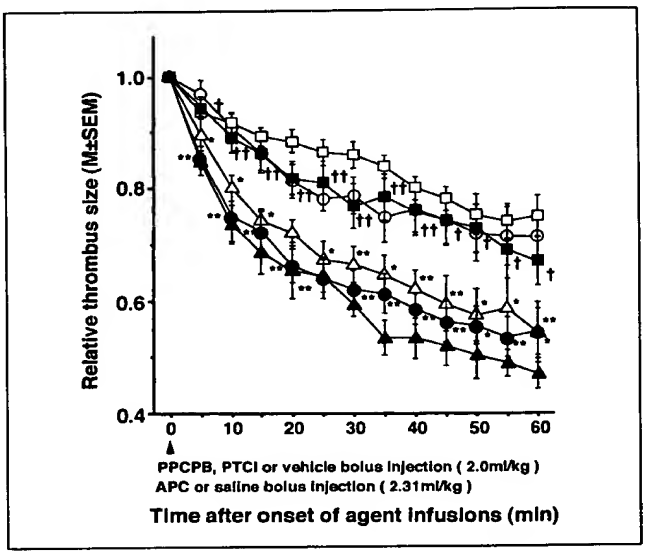

Fig. 3 Inhibition of APC-induced thrombolysis by PPCPB and enhancement of the endogenous thrombolysis by PTCI. ●: APC (2.0 mg/kg/h) + vehicle; □: saline + PPCPB (3.5 mg/kg); △: saline + PTCI (5.0 mg/kg); ■: APC + PPCPB; ▲: APC + PTCI; ○: saline + vehicle (control). n = 6~8 rats in each group. *: $p < 0.05$, **: $p < 0.01$ versus control and †: $p < 0.05$, ††: $p < 0.01$ versus APC + vehicle group in each time interval

Administration of APC alone also enhanced endogenous thrombolysis. APC is known to degrade coagulation factors Va and VIIIa and thus limits the autocatalytic production of thrombin in factor XIa-mediated coagulation pathways (29, 30). In the current studies, it seems likely, therefore, that APC depressed thrombin generation and hence restricted the generation of TAFIa. Alternatively, other reports have shown that APC forms an equimolar complex with plasminogen activator inhibitor-1 (PAI-1), and this could have mediated increased endogenous, PA-induced thrombolysis (31). APC demonstrates species specificity toward to PAI-1 (32). However, it is uncertain whether the currently used human APC can bind to rat PAI-1. Further, plasmin is also thought to be an activator of TAFI (33). The full physiological significance of TAFI activation remains to be clarified. Nevertheless, the present study demonstrated that endogenous fibrinolysis or thrombolysis was enhanced when thrombin or thrombin generation was inhibited, and that TAFI played a significant role in these mechanisms. The pathophysiological relevance of endogenous fibrinolysis regulated by thrombin is unclear at present but our findings indicate that inhibition of thrombin-related responses may be clinically important in the prevention and treatment of thrombosis not only by limiting thrombus formation but also by enhancing thrombus dissolution. Assessment of endogenous thrombolysis in human cardiovascular disorders may help to clarify the role of TAFI in maintaining vascular patency *in vivo*.

In conclusion, we have established a simple, highly sensitive and reproducible *in vivo* model of arterial thrombolysis using rat mesenteric arterioles. Our results strongly suggest that the thrombin-TAFIa system plays a significant role in the regulation of thrombolysis or fibrinolysis *in vivo* as suggested by earlier *in vitro* studies.

Acknowledgements

We would like to thank Dr. Y. Sasaki and Ms. T. Taka, Laboratory of Physiology, Faculty of Nutrition and High Technology Research Centre, Kobe Gakuin University, Kobe, Japan, for their useful discussion.

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ORIGINAL ARTICLE

Factor XII-dependent increases in thrombin activity induce carboxypeptidase-mediated attenuation of pharmacological fibrinolysis

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Summary. Activation of the contact system in patients treated with fibrinolytic agents may be an important source of thrombin that activates thrombin-activated fibrinolysis inhibitor (TAFI) and attenuates fibrinolysis. Factor (F)XIIa in plasma increased 2-fold over 60 min in patients given either tissue plasminogen activator (t-PA) or streptokinase (SK). To determine whether FXIIa-mediated generation of thrombin and activated TAFI (TAFIa) attenuates fibrinolysis *in vitro*, plasma clots were incubated with SK (250 U mL^{-1}) or t-PA (2.5 g mL^{-1}) and the rate of lysis was measured. Plasma FXIIa impaired lysis judging from marked acceleration when 2.5 μM corn trypsin inhibitor were added (lysis increased by 172 ± 144% for SK and 40 ± 31% for t-PA vs. no inhibitor, $n = 16$, $P < 0.01$). Moreover, inhibition of thrombin with hirudin and TAFIa with carboxypeptidase inhibitor accelerated lysis. We conclude that activation of FXII increases thrombin generation, which promotes TAFIa-mediated attenuation of fibrinolysis.

Keywords: carboxypeptidase, factor XII, thrombin-activated fibrinolysis inhibitor, thrombolysis.

Introduction

It is well known that increases in thrombin activity occur in patients with myocardial infarction treated with fibrinolytic agents, judging from increases in plasma concentrations of markers of thrombin activity such as fibrinopeptide A (FPA) [1–4]. Simultaneous increases in plasma concentrations of prothrombin fragment 1.2 and thrombin–antithrombin III complexes in such patients indicate that increases in thrombin activity are attributable, at least in part, to increased prothrombin activation [5,6]. Others and we have found evidence for several mechanisms by which pharmacological fibrinolysis may potentiate activation of the coagulation system including plasmin-mediated activation of factor (F)XII and factor (F)V [7–9], potentiation of platelet-dependent prothrombin activation, and exposure of thrombus-associated factor (F)Xa/FVa and thrombin during clot lysis [10–12]. Based on analysis of plasma markers in patients given fibrinolytic agents, increases in thrombin activity may be a determinant of the failure of coronary thrombolysis [1,6,13]. Moreover, based on studies in both experimental animals and patients, it appears that thrombin inhibitors accelerate thrombolysis and improve the clinical outcome [14,15].

A potential mechanism for improved thrombolysis in the presence of thrombin inhibitors is reduced activation of a thrombin-activatable inhibitor of fibrinolysis (thrombin-activated fibrinolysis inhibitor, TAFI) [16–18]. TAFI has been identified as plasma carboxypeptidase B, which upon activation by thrombin (to TAFIa) cleaves lysine and arginine from fibrin that serve to potentiate plasminogen activation on the fibrin surface [16–19]. Inhibition of plasma carboxypeptidase activity or activation of TAFI have been shown to potentiate clot lysis *in vitro* [16]. Inhibition of plasma carboxypeptidase activity was shown also to accelerate arterial and venous fibrinolysis induced by plasminogen activators in experimental animals [20–23]. However, the mechanisms by which TAFI is activated in response to pharmacological fibrinolysis have not been defined. We hypothesized that plasmin-mediated activation of the contact system in patients treated with fibrinolytic agents may be an important mechanism for thrombin generation that leads to activation of TAFI and subsequent attenuation of fibrinolysis. Accordingly, the objectives of this study were to characterize further FXII activation in patients treated with tissue plasminogen activator (t-PA) and streptokinase (SK), and to determine whether FXIIa results in thrombin-mediated activation of TAFI that could attenuate the rate of clot lysis *in vitro*.

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Materials and methods

Measurement of FXIIa in plasma from patients treated with t-PA or SK

Venous blood samples were withdrawn from patients with informed consent before and 60 min after treatment with 100 mg t-PA ($n = 9$) or 1.5 million U SK ($n = 29$) into tubes containing PPACK, EDTA, and aprotinin [24]. Samples were centrifuged and the plasma was stored at −70 °C. FXIIa in plasma was assayed with use of an antihuman FXIIa monoclonal antibody (Shield Diagnostics, Dundee, UK) as described [25]. Briefly, aliquots (100 μL) of plasma or FXIIa standards were incubated for 60 min in a 96-well microtiter plate coated with the antibody. The plate was drained, the wells washed five times with borate buffer, and solution (100 μL) containing an alkaline phosphatase-labeled polyclonal sheep antihuman FXIIa antibody was incubated in each well for 60 min. The plate was washed with buffer and a substrate solution (100 μL) containing phenolphthalein monophosphate (0.1%, w/v) was added to the wells and incubated for 15 min. The reaction was stopped by addition of 100 μL of 0.4 M sodium carbonate buffer containing 0.1 M 3-(cyclohexylamine)-1-propane sulfonic acid, 0.1 M EDTA and 0.4 M sodium hydroxide. The absorbance was measured at 550 nm in an automated microtiter plate reader (ThermoMax; Molecular Devices, Sunnyvale, CA, USA). Results from duplicate assays of plasma samples were compared with the FXIIa standard curve.

Clot lysis in response to pharmacological concentrations of fibrinolytic agents

Materials Citrated human plasma was purchased from the American Red Cross (St Louis, MO, USA). Plasma from at least four donors was pooled, aliquoted and frozen at −70 °C until use. Plasma from patients with congenital fibrinogen or FXII deficiency was purchased from George King Biomedical (Overland Park, KS, USA). Human prothrombin was isolated from pooled, citrated plasma by adsorption to sulfated dextran beads followed by ammonium sulfate elution and DE-52 cellulose ion-exchange chromatography as described previously [26]. The recovered prothrombin was >99% free of contaminating proteins. Thrombin was prepared by activation of prothrombin with Taipan snake venom followed by purification with Mono-S chromatography. SK was purchased from Behringwerkeage (Marburg/Lahn, Germany), urokinase (UK) was purchased from Abbott Labs (North Chicago, IL, USA), and recombinant t-PA was a gift from Genentech (South San Francisco, CA, USA). Recombinant desulfatohirudin (hirudin) was a gift from Ciba-Geigy (Basel, Switzerland) and recombinant tick anticoagulant peptide (TAP) was a gift from Dr G. Vlasuk (Corvas, San Diego, CA, USA). Corn trypsin inhibitor (CTI) was purchased from Enzyme Research Labs (South Bend, IN, USA), unfractionated heparin was purchased from Elkins-Sinn (Cherry Hill, NJ, USA), and the carboxypeptidase inhibitor, DL-2-mercaptomethyl-3-guanidinoethylthiopropanoic acid, also known as Plummer's Inhibitor, was purchased from Calbiochem (La Jolla, CA, USA). A monoclonal antibody that inhibits thrombin-mediated activation of TAFI was a generous gift from Dr M. Nesheim [27]. Purified TAFI was purchased from Haematologic Technologies (Essex Junction, VT, USA).

Clot lysis assay Plasma clots were formed by addition of 75 μL of citrated, pooled plasma to 96-well microtiter plates together with 75 μL of 0.1 M Tris buffer containing 1 U mL^{-1} thrombin and 25 mM $CaCl_2$ (final concentrations). The solution was incubated and absorbance was monitored at 405 nm. Absorbance typically increased rapidly during clot formation and was stable within 45 min. After clot formation, 150 μL of undiluted, pooled or factor-deficient plasma together with plasminogen activators and inhibitors, in some cases, were added to the wells and recalcified (25 mM final concentration). Based on the results of previous studies and preliminary experiments, plasminogen activators were added in the following concentrations: 1000 U mL^{-1} UK, 250 U mL^{-1} SK, or 2.5 μg mL^{-1} t-PA. These concentrations are similar to the low range of plasma concentrations achieved during treatment of patients with myocardial infarction. Plasminogen activators and inhibitors were delivered to the wells simultaneously and absorbance was monitored continuously for 120 min at 37 °C. The linear portion of the decrease in absorbance was used to calculate the rate of clot lysis. The baseline rate for each plasminogen activator was compared with lysis rates in the presence of coagulation and carboxypeptidase inhibitors in subsequent experiments.

Statistical analysis

All data are presented as the mean ± SD. Linear rates of clot lysis between conditions were analyzed by factorial analysis of variance (ANOVA) with Statview 4.5 software (SAS Institute, Cary, NC, USA) on a Macintosh power PC. *Post hoc* analysis was with Scheffe's *F*-test. A value of $P \leq 0.05$ was considered significant.

Results

FXII activation in patients treated with t-PA or SK

Concentrations of FXIIa in plasma increased significantly after administration of either 100 mg t-PA or 1.5 million U SK in nearly all of the patients tested (Fig. 1a,b).

Fibrin formation and lysis in response to pharmacological plasminogen activation

To determine whether activation of the contact system of coagulation induces thrombin-mediated activation of TAFI resulting in attenuation of clot lysis, plasma clots were incubated with recalcified citrated plasma containing either

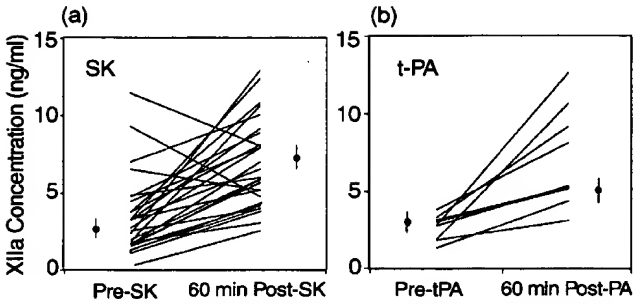

Fig. 1. Concentrations of factor (F)XIIa in plasma from patients with acute myocardial infarction treated with either 1 million U streptokinase (SK) over 60 min ($n = 20$) (a) or 100 mg tissue plasminogen activator (t-PA) over 90 min ($n = 9$) (b). Concentrations of FXIIa increased significantly after infusion of either SK or t-PA ($P = 0.002$ for SK and $P = 0.0026$ for t-PA compared with preinfusion values).

250 U mL^{-1} SK, 2.5 μg mL^{-1} t-PA, or 1000 U mL^{-1} UK. Addition of plasminogen activators resulted in a transient increase in absorbance, followed by a linear decrease in absorbance as clot lysis was induced (Fig. 2). The transient increase in absorbance was probably attributable to fibrin formation, judging from the lack of an increase when plasma from a patient with congenital fibrinogen deficiency containing 250 U SK was added to the clots (Fig. 2a).

Effects of inhibition of thrombin and FXa on the rates of clot lysis

To confirm that inhibition of thrombin accelerated clot lysis, recalcified citrated plasma containing either t-PA, UK, or SK, and 3 μM hirudin was added to the clots, and the rates of lysis compared with those in the absence of hirudin. Hirudin at a concentration of 3 μM accelerated the rate of clot lysis for each of the plasminogen activators, with the greatest effect observed for SK (Figs 2b and 3a). Nearly identical results were obtained when plasminogen activators were incubated with clots in the presence of 5 μM TAP, a specific inhibitor of FXa (Fig. 3b).

Effect of FXII on the rate of clot lysis

The role of FXII in thrombin-mediated attenuation of clot lysis was characterized by incubating normal plasma clots with recalcified, citrated FXII-deficient plasma containing 250 U mL^{-1} SK either with or without the addition of 75 μg mL^{-1} purified human FXII. Clot lysis was accelerated by 61 ± 35% in FXII-deficient plasma compared with lysis in response to the same concentration of SK in FXII-deficient plasma repleted with FXII ($P = 0.0002$) (Fig. 4a).

The importance of activation of FXII in mediating attenuation of clot lysis was confirmed by incubating plasma clots with 2.5 μg mL^{-1} t-PA or 250 U mL^{-1} SK in recalcified, pooled, citrated plasma in the presence or absence of 2.5 μM CTI, a specific inhibitor of FXIIa. Clot lysis in the presence of CTI was accelerated by 172 ± 144% for SK and 40 ± 31% for t-PA compared with lysis rates in the absence of CTI ($P < 0.01$ for both activators compared with rates without CTI) (Fig. 4b). Acceleration of lysis was not attributable to direct inhibition of TAFIa by CTI, judging from the lack of inhibition of the activity of purified TAFIa against the synthetic substrate for carboxypeptidase, hippuryl-L-arginine (data not shown). Thus, either deficiency of FXII or specific inhibition of FXIIa accelerated clot lysis induced by t-PA and SK.

Role of carboxypeptidase in attenuating clot lysis

To confirm the role of carboxypeptidase activity in attenuating the rate of clot lysis induced by pharmacological plasminogen activation, plasminogen activators were incubated with clots in the presence of DL-2-mercaptomethyl-3-guanidinoethylthiopropanoic acid, an inhibitor of both plasma carboxypeptidase

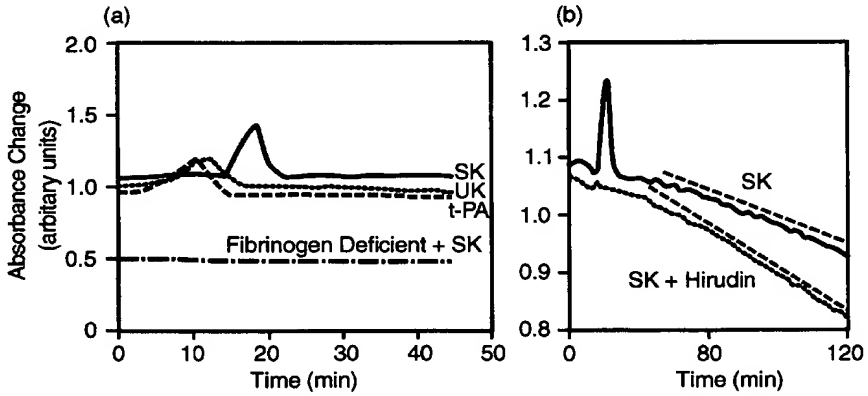

Fig. 2. Representative changes in absorbance after addition to plasma clots of recalcified, pooled, citrated plasma and plasminogen activators [streptokinase (SK) 250 U mL^{-1}; urokinase (UK) 1000 U mL^{-1}; tissue plasminogen activator (t-PA) 2.5 μg mL^{-1}]. (a) Absorbance increased early because of increased fibrin formation, judging from the lack of increase when SK was added to fibrinogen-deficient plasma. (b) After 40–50 min, absorbance decreased consistent with clot lysis. The linear portion of the profiles shown by the dashed lines was used for lysis rate calculations. Lysis with SK was accelerated by concurrent incubation with 1 μm hirudin, which also eliminated the initial transient increase in absorbance attributable to fibrin formation.

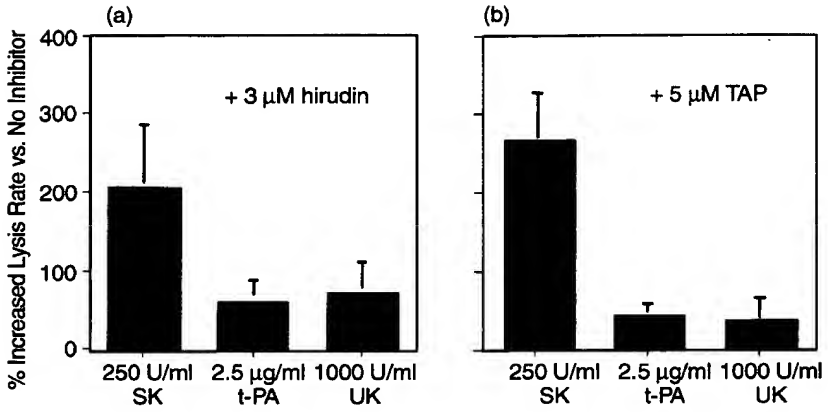

Fig. 3. Acceleration of clot lysis rates during incubation with plasminogen activators in the presence of the thrombin inhibitor, hirudin (3 μm) (a), or factor Xa inhibitor, tick anticoagulant peptide (TAP, 5 μm) (b). Data are expressed as the percent increase in the rate of clot lysis compared with no inhibitor as a control. Incubation of clots with either hirudin or TAP significantly accelerated lysis for each of the activators ($P < 0.01$) ($n = 16$).

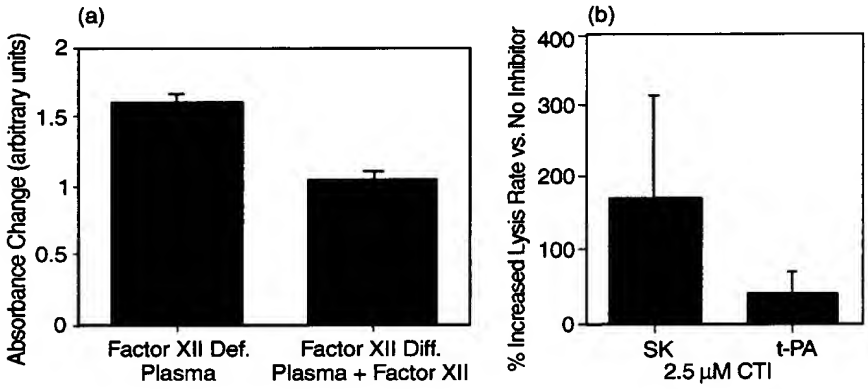

Fig. 4. Changes in absorbance during clot lysis in recalcified, citrated, factor (F)XIIa-deficient and repleted plasma (a) and the effect on the rate of lysis after addition of corn trypsin inhibitor (CTI, 2.5 μm) (b). (a) Lysis induced with 250 U mL^{-1} streptokinase (SK) was significantly attenuated when FXII-deficient plasma was repleted with FXII ($P = 0.0002$). (b) Inhibition of FXIIa with CTI accelerated the rate of clot lysis induced with either 250 U mL^{-1} SK or 2.5 μg mL^{-1} tissue plasminogen activator (t-PA) ($P < 0.01$ for the increase in clot lysis with CTI rates compared with without CTI) ($n = 16$).

N and B [28]. Incubation with the inhibitor accelerated clot lysis by 189 ± 128% for SK and 67 ± 37% for t-PA ($P < 0.01$ for both compared with rates without inhibitor). Accelerated lysis was not due to inhibition of thrombin activity, judging from the lack of attenuation of thrombin amidolytic activity against the synthetic substrate H-D phe-pip-arg-p-nitroaniline (S-2238; Chromogenics, Diapharma Group Inc., West Chester, OH, USA) (data not shown).

To confirm that the inducible carboxypeptidase activity was attributable to TAFI, the rate of clot lysis induced by SK was measured in the presence of a monoclonal antibody that inhibits activation of TAFI, but not the activity of TAFIa. The anti-TAFI monoclonal antibody accelerated the rate of clot lysis induced by SK in a concentration-dependent manner, with the maximal effect at 0.3 μg mL^{-1} of antibody ($P < 0.01$ compared with no antibody, Fig. 5a,b).

In contrast to the results when clot lysis was induced in recalcified, pooled, citrated plasma, neither DL-2-mercaptomethyl-3-guanidinoethylthiopropanoic acid nor the anti-TAFI antibody increased the rate of clot lysis significantly for SK in FXII-deficient plasma (data not shown).

Discussion

The results of this study show that thrombin elaboration in response to pharmacological fibrinolysis may attenuate the rate of clot lysis by inducing activation of TAFI. In addition, we have provided further evidence that thrombin elaboration in response to administration of fibrinolytic agents may be mediated by FXII-dependent activation of the contact system. This latter conclusion is consistent with our previous results showing direct activation of FXII by plasmin in plasma and in whole

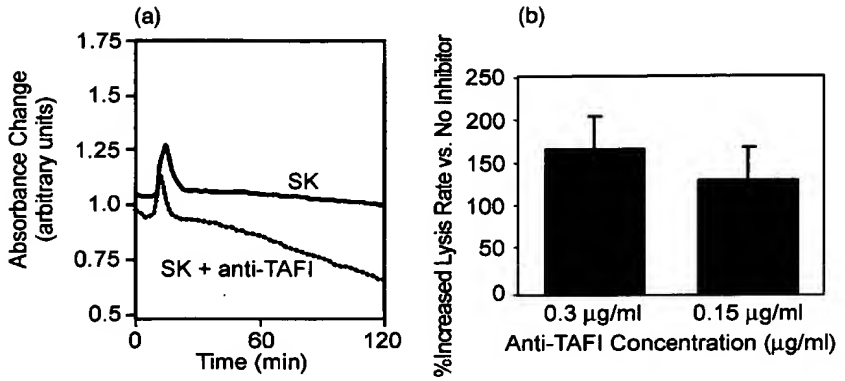

Fig. 5. Acceleration of clot lysis by inhibition of thrombin-activated fibrinolysis inhibitor (TAFI) activation. (a) The absorbance change induced with streptokinase (SK) (250 U mL^{-1}) was increased in the presence of anti-TAFI monoclonal antibodies. Note in contrast to the results with hirudin (Fig. 2b), anti-TAFI antibody accelerated lysis without inhibiting initial increases in clot formation induced by thrombin elaboration. (b) Accelerated lysis with anti-TAFI monoclonal antibody was concentration dependent ($P < 0.01$) for both concentrations of anti-TAFI compared with rates in the absence of antibody ($n = 8$).

blood resulting in thrombin elaboration [7]. The results of the present study suggest that acceleration of coronary thrombolysis documented previously in experimental animals during specific inhibition of FXa and thrombin is attributable, in part, to decreased activation of TAFI [14,29–31]. This conclusion is supported by reports that fibrinolysis of either arterial or venous thrombi was accelerated when inhibitors of carboxypeptidase were administered conjunctively with plasminogen activators [20–23]. The critical questions are whether the FXII-dependent mechanism described in this study plays an important role in thrombin elaboration in patients treated with fibrinolytic agents, and what the implications of activation of TAFI are on the success of thrombolytic therapy.

Direct procoagulant effects of plasminogen activators on coagulation proteins and platelets have been described [5,7,8,32,33], as well as the potential for fibrinolysis to expose procoagulants that may accelerate activation of the coagulation system [10–12]. We have previously documented increases in plasma concentrations of FPA within 30 min of the initiation of SK infusion in patients, and marked attenuation of the increase by coadministration of heparin suggesting direct activation of the coagulation system [1,2]. In subsequent *in vitro* studies, t-PA, UK, and SK at concentrations similar to those achieved in patients were shown to induce thrombin elaboration directly in non-anticoagulated whole blood and plasma [32,34]. More recently, activation of the coagulation system in response to incubation with fibrinolytic agents was shown to be dependent on FXII, and could be completely inhibited in non-anticoagulated whole blood by inhibition of FXIIa [7]. The results of the present study provide further support for this mechanism, by confirming the activation of FXII in patients treated with SK or t-PA (Fig. 1). Our findings are similar to those of Coppola *et al.* [35], who also documented marked increases in FXIIa levels after a 90-min infusion of SK or t-PA in patients treated for acute myocardial infarction. The procoagulant effects of pharmacological plasminogen activation are transient, as noted in the present study, because plasmin degrades and inactivates coagulation factors, such as high-molecular-weight kininogen, and FV and FVIII [8,36,37]. Our results suggest that the early transient elaboration of thrombin may have important consequences on the initial rate of clot lysis, by inducing activation of TAFI.

Activation of TAFI by factor XIa has also been shown to attenuate physiological jugular vein clot lysis in rabbits [38]. Our results suggest that activation of TAFI in response to pharmacological plasminogen activation is primarily mediated by thrombin elaboration induced by activation of the contact system. The finding that anti-TAFI antibody did not potentiate the rate of clot lysis induced by SK in FXII-deficient plasma compared with pooled plasma is consistent with this hypothesis.

Clinical data regarding the importance of either thrombin elaboration or activation of TAFI on the outcome of thrombolytic therapy for acute myocardial infarction are lacking. Nonetheless, the results of studies based on plasma markers have shown that the extent of thrombin elaboration and activity is significantly greater in patients with myocardial infarction treated with t-PA who fail to achieve TIMI-3 grade coronary blood flow by 90 min [39]. Similarly, direct inhibition of thrombin with hirudin or hirulog has been shown to increase the rate of TIMI-3 patency at 90 min compared with heparin in patients treated with t-PA or SK [40,41]. Despite these encouraging data, a large-scale clinical trial failed to show a survival advantage for conjunctive therapy with hirudin compared with heparin in patients treated with these activators [42]. Although there appeared to be only marginal benefit for the conjunctive therapy with hirudin in another large-scale trial [43], reassessment of the data indicated that direct thrombin inhibition improved outcome in patients treated with SK, but not t-PA [15]. An important feature of the clinical trials is that the required randomization of the anticoagulant regimen delayed initiation of hirudin and heparin to a time when substantial thrombin elaboration had already occurred [2]. The results of this study and the extensive characterization of the kinetics of activation of TAFI by thrombin reported previously

[10] underscores the fact that thrombin, particularly in the presence of thrombomodulin *in vivo* [18], would rapidly induce activation of TAFI. Thus, the results of previous clinical studies may underestimate the potential impact of acceleration of thrombolysis with either earlier, complete thrombin inhibition, or direct inhibition of TAFIa.

In summary, our results provide additional support for the hypothesis that the success of pharmacological fibrinolysis is determined by a dynamic balance between procoagulant and fibrinolytic activity. The relevant mechanisms involved in determining the procoagulant and fibrinolytic activity will vary depending on the fibrinolytic agent used. The results of this and our previous studies suggest that fibrinolytic agents that induce free plasmin activity (i.e. not fibrin-selective) have a greater propensity to activate the contact system of coagulation and indirectly attenuate the rate of clot lysis by activating TAFI. Whether more fibrin selective agents or the use of novel inhibitors of the coagulation system will improve the outcome of fibrinolytic therapy remains to be determined.

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